

Express Mail Label No.: EL932680304US  
Attorney Docket No.: 46342/57113

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**  
**NEW UTILITY PATENT APPLICATION**

Entitled: SCREENING METHOD

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## SPECIFICATION

## SCREENING METHOD

## FIELD OF THE INVENTION

The present invention relates to a method of efficiently screening a compound which promotes or inhibits a function of an orphan receptor protein by screening a highly concentrated test compound using cells capable of expressing an orphan receptor as an index and using a common structure of test compounds having a function of an agonist, and also relates to a method of identifying (endogenous) ligands of the orphan protein receptor.

## BACKGROUND ART

Physiologically active substances such as various hormones and neurotransmitters regulate the biological function via specific receptor proteins present on cell membranes. It has been an important means for the pharmaceutical research and development to search substances which fills, increases and inhibits the function of physiological activity. It is quite important to understand properties of receptor molecules, which work as a point of action in the process. In recent years, sharing the benefit from the development in molecular biology, it became possible to analyze receptors for physiological active substances at a molecular level. Among such receptor molecules, a series of receptors possess a common structure containing seven transmembrane domains and are thus called G protein-coupled receptors or seven-transmembrane receptors (7TMR). These receptor proteins are coupled with intracellular signal transduction system through a GTP-binding protein (G

protein), and thus are referred to as G-protein coupled receptors. Seven-transmembrane receptor ligands include various things such as proteins, peptides, amines, amino acids, nucleotides, eicosanoids, phospholipids, scent substances, light and etc.

In recent years, because of the development of analysis technologies for genomes or cDNAs, many genes encoding receptors have been reported. Some of these genes are estimated to belong to the seven-transmembrane receptor family in view of their characteristics of the sequence. However, since the corresponding ligands have not been known yet, they are called an orphan receptor. If a ligand of an orphan receptor can be identified, it is expected to clarify novel physiological functions or pathologies modulated by the receptor and the ligand. Therefore, screening of orphan receptor ligands is considered to be an important means for discovering a new target in pharmaceutical development. There is such an example that orphanin FQ/nociceptin (Meunier, J. -C. Nature 393:211-212, 1998) was successfully identified as an endogenous ligand, by examining known ligands or related substances for those an orphan receptor showing remarkable similarities, to some extent, to the ligand known receptors. However, there is limitation in estimating a structure of ligand only from the structural similarities to known ligands or related substances. In most of cases, an activation of signal transmitters of cells capable of expressing orphan receptor proteins as an index, an identification of endogenous ligands was taking place by purification (Sakurai. T. et al. Cell 92: 573-585, 1998; Hinuma, S, et al. Nature 393:272-276, 1998; Tatemoto, K. et al. Biochem. Biophys. Res. Commun. 251: 471-476, 1998). However, the signal transmission system via a seven-

transmembrane receptor is not a single one. Thus, to detect activities derived from endogenous ligands, it is necessary to conduct many assay lines for screening in parallel. The seven-transmembrane receptor ligands include variety of things. It is not easy to select samples rationally for ligand candidates to be tested in assays.

To establish an efficient and reliable method for screening compounds and their salts, which promote or inhibit a function of an orphan receptor protein or a method of identifying (endogenous) ligands, is considered to be the major issue.

#### DISCLOSURE OF THE INVENTION

In order to solve above problems, the present inventors have made extensive studies and as a result, found that screening of a compound which promotes or inhibits a function of an orphan receptor protein or its salt can be effectively carried out by (i) measuring a cell stimulating activity to be measured when a test compound (preferably, highly concentrated) is brought in contact with cells capable of expressing an orphan receptor protein or its cell membranes fractions, and a cell stimulating activity to be measured when a test compound is brought in contact with expression cells which are not capable of expressing an orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of test compounds having an agonist activity, and

(iii) ① comparing a cell stimulating activity to be measured when a ligand candidate having said common

structure is brought in contact with said cells capable of expressing an orphan receptor protein or its cell membrane fractions with a cell stimulating activity to be measured when a candidate compound substance which promotes or inhibits the function of said orphan receptor protein is brought in contact with said cells capable of expressing an orphan receptor protein or its cell membrane fractions, and ② measuring the amount of specific binding between said orphan receptor protein and a compound which promotes or inhibits the function of said orphan receptor protein,. Based on these findings, the present inventors made continued extensive studies and as a result, accomplished the present invention. Thus, the present invention relates to, for example, the following:

(1) A method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein, comprising:

(i) measuring a cell stimulating activity to be measured when a test compound is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, and a cell stimulating activity to be measured when the test compound is brought in contact with cells which are not capable of expressing the orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a common structure of test compound(s) having an agonist activity,

(iii) ① comparing a cell stimulating activity to be measured when a ligand candidate compound which is selected by considering a common structure of said compounds having an agonist activity is brought in contact with said cells capable of expressing the

orphan receptor or its cell membrane fractions, and a cell stimulating activity to be measured when a test compound is brought in contact with said cells capable of expressing the orphan receptor or its cell membrane fractions, and ② measuring the amount of specific binding between said orphan receptor protein and the test compound.

(2) A compound or a salt thereof obtainable by the screening method according to the screening method described in above (1), and

(3) A method of identifying a ligand or its subtypes, comprising:

(i) measuring a cell stimulating activity to be measured when a test compound is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, and a cell stimulating activity to be measured when a test compound is brought in contact with cells which are not capable of expressing an orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound, to identify a compound(s) having an agonist activity, and

(iii) measuring amount of specific binding between said orphan receptor protein and a ligand candidate compound which is selected by considering a common structure of the compounds having an agonist activity.

More specifically, the present invention relates to, for example, the following:

(4) A method of screening a compound or its salt, which promotes or inhibits a function of an orphan receptor protein, comprising:

(i) measuring a cell stimulating activity to be measured when test compound (a) is brought in contact with cells capable of expressing an orphan receptor or

its cell membrane fractions, and a cell stimulating activity to be measured when test compound (a) is brought in contact with cells which are not capable of expressing the orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound (a), to identify compounds having an agonist activity,

(iii) ① comparing a cell stimulating activity to be measured when a ligand candidate compound which is selected by considering a common structure of said compounds having an agonist activity is brought in contact with said cells capable of expressing the orphan receptor or its cell membrane fractions, and a cell stimulating activity to be measured when test compound (b) is brought in contact with said cells capable of expressing the orphan receptor or its cell membrane fractions, and ② measuring amount of specific binding between said orphan receptor protein and test compound (b),

(5) A compound or a salt thereof obtainable by the screening method according to the screening method described in above (4), and

(6) A method of identifying a ligand or its subtypes, comprising:

(i) measuring a cell stimulating activity to be measured when test compound (a) is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, and a cell stimulating activity to be measured when test compound (a) is brought in contact with cells which are not capable of expressing the orphan receptor or its cell membrane fractions, respectively,

(ii) comparing the cell stimulating activities thus measured for each test compound (a), to identify compounds having an agonist activity, and

(iii) measuring amount of specific binding between said orphan receptor protein and a ligand candidate compound which is selected by considering a common structure of the compounds having an agonist activity.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the comparison of commonness of amino acids in EXAMPLE 1. A common amino acid residues are shown with squares surrounding them. Since phenyl alanin (F) and tyrosine (Y) show similar steric structure, they are also indicated with squares surrounding them as well.

#### BEST MODE FOR CARRYING OUT THE INVENTION

##### (A) The orphan receptor protein:

In this specification, "orphan receptor proteins" means proteins the ligand of which has not been known, including the both publicly known and unknown ones.

Examples of orphan receptor proteins are; FM-3 receptor protein (Tan, C. P. et al, Genomics 52,223-229, 1998) used in Example 1 described later, mas receptor protein (Young D. et. al., Proc. Natl. Acad. Sci. USA, 85,5339-5342, 1988), etc. Moreover, they are listed in the Swiss-plot database for orphan receptors.

The orphan receptor proteins useful in the present invention may form salts. The salts of "orphan receptor proteins" may be salts with physiologically acceptable bases (e.g., alkali metals) or salts with physiologically acceptable acids, especially physiologically acceptable acid addition salts. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric



acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

The DNA encoding an orphan receptor protein may be any DNA comprising DNA encoding an orphan receptor protein. It could be a genome DNA, genome DNA library, a cDNA derived from any cells of human and other warm-blooded animals (e.g., guinea pig, rat, mouse, rabbit, swine, sheep, bovine, monkey, etc.) such as retina cells, liver cells, splenocytes, nerve cells, glial cells,  $\beta$  cells of pancreas, bone marrow cells, mesangial cells, Langerhans' cells, epidermic cells, epithelial cells, endothelial cells, fibroblasts, fibrocytes, myocytes, fat cells, immune cells (e.g., macrophage, T cells, B cells, natural killer cells, mast cells, neutrophil, basophil, eosinophil, monocyte), megakaryocyte, synovial cells, chondrocytes, bone cells, osteoblasts, osteoclasts, mammary gland cells, hepatocytes or interstitial cells, the corresponding precursor cells, stem cells, cancer cells, etc., hemocyte type cells, or any tissues where such cells are present, e.g., brain or any region of the brain (e.g., olfactory bulb, amygdaloid nucleus, basal ganglia, hippocampus, thalamus, hypothalamus, subthalamus, cerebral cortex, medulla oblongata, cerebellum, occipital pole, frontal lobe, temporal lobe, putamen, caudate nucleus, corpus callosum, substantia nigra), spinal cord, hypophysis, stomach, pancreas, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract (e.g., large intestine and small intestine), blood vessel, heart, thymus, spleen,

submandibular gland, peripheral blood, peripheral blood cells, prostate, testis, ovary, placenta, uterus, bone, joint, skeletal muscle, hemocyte type cells or its cultured cell (e.g., MEL, M1, CTLL-2, HT-2, WEHI-3, HL-60, JOSK-1, K562, ML-1, MOLT-3, MOLT-4, MOLT-10, CCRF-CEM, TALL-1, Jurkat, CCRT-HSB-2, KE-37, SKW-3, HUT-78, HUT-102, H9, U-937, THP-1, HEL, JK-1, CMK, KO-812, MEG-01) etc. from human and other mammals (e.g., guinea pigs, rats, mice, rabbits, swine, sheep, bovine, monkeys, etc.). The receptor protein may also be a cDNA library derived from said tissues, cells or a synthetic protein. The vector to be used for the library may be any of bacteriophage, plasmid, cosmid and phagemid. The DNA may also be directly amplified by reverse transcriptase polymerase chain reaction (hereinafter abbreviated as RT-PCR) using the total RNA or mRNA fraction prepared from the cells and tissues.

The DNA encoding an orphan receptor protein can be prepared according to the following genetic engineering method.

For cloning of the DNA that completely encodes the orphan receptor protein, the DNA may be either amplified by PCR using synthetic DNA primers containing a part of the base sequence of the orphan receptor protein, or the DNA inserted into an appropriate vector can be selected by hybridization with a labeled DNA fragment or synthetic DNA that encodes a part or entire region of the receptor protein of the present invention. The hybridization can be carried out, for example, according to the method described in Molecular Cloning, 2nd, J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989. The hybridization may also be performed using commercially available library in accordance with the protocol described in the attached instructions.

The cloned DNA encoding an orphan receptor protein can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may contain ATG as a translation initiation codon at the 5' end thereof and may further contain TAA, TGA or TAG as a translation termination codon at the 3' end thereof. These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

(B) The cells capable of expressing an orphan receptor protein and its cell membrane fractions or orphan receptor protein expression cells and its cell membrane fractions:

In this specification, "cells capable of expressing an orphan receptor protein" means host cells expressing the above orphan receptor protein (A). As to the method of expressing an orphan receptor protein at a host cell, it can be expressed using the DNA encoding the above orphan receptor protein (A) according to the following method.

That is, the expression vector for the receptor protein can be prepared, for example, by (a) excising the desired DNA fragment from the DNA encoding the receptor protein of the present invention, and then (b) ligating the DNA fragment with an appropriate expression vector downstream a promoter in the vector.

Examples of the vector include plasmids derived from *E. coli* (e.g., pBR322, pBR325, pUC12, pUC13), plasmids derived from *Bacillus subtilis* (e.g., pUB110, pTP5, pC194), plasmids derived from yeast (e.g., pSH19, pSH15), bacteriophages such as  $\lambda$  phage, etc., animal viruses such as retrovirus, vaccinia virus, baculovirus, etc. The promoter used in the present invention may be

any promoter if it matches well with a host to be used for gene expression.

In the case of using animal cells as the host, examples of the promoter include SV40-derived promoter, retrovirus promoter, metallothionein promoter, heat shock promoter, a cytomegalovirus promoter, SR $\alpha$  promoter, etc.

Where the host is bacteria of the genus *Escherichia*, preferred examples of the promoter include Trp promoter, T-7 promoter, lac promoter, recA promoter,  $\lambda$ PL promoter, lpp promoter, etc. In the case of using bacteria of the genus *Bacillus* as the host, preferred examples of the promoter are SP01 promoter, SP02 promoter and penP promoter. When yeast is used as the host, preferred examples of the promoter are PH05 promoter, PGK promoter, GAP promoter, ADH1 promoter, GAL promoter. When insect cells are used as the host, preferred examples of the promoter include polyhedrin promoter and P10 promoter. In addition, in the present invention, to measure a cell stimulating activity mediated by an orphan receptor protein, preferred hosts are animal cells and insect cells.

In addition, if desired, the expression vector may further optionally contain an enhancer, a splicing signal, a poly A addition signal, a selection marker, SV40 replication origin (hereinafter sometimes abbreviated as SV40ori) etc. Examples of the selection marker include dihydrofolate reductase (hereinafter sometimes abbreviated as dhfr) gene [methotrexate (MTX) resistance], ampicillin resistant gene (hereinafter sometimes abbreviated as Amp<sup>r</sup>), neomycin resistant gene (hereinafter sometimes abbreviated as Neo<sup>r</sup>, G418 resistance), etc. In particular, when DHFR gene is used as the selection marker in CHO (dhfr<sup>-</sup>) cells, selection can also be made on thymidine free media.

If necessary and desired, a signal sequence that matches with a host is added to the N-terminus of the receptor protein of the present invention. Examples of the signal sequence that can be used are Pho A signal sequence, OmpA signal sequence, etc. in the case of using bacteria of the genus *Escherichia* as the host;  $\alpha$ -amylase signal sequence, subtilisin signal sequence, etc. in the case of using bacteria of the genus *Bacillus* as the host; mating factor (MF $\alpha$ ) signal sequence, invertase signal sequence, etc. in the case of using yeast as the host; and insulin signal sequence,  $\alpha$ -interferon signal sequence, antibody molecule signal sequence, etc. in the case of using animal cells as the host, respectively.

Using the vector containing the DNA encoding an orphan receptor protein thus constructed, transformants can be manufactured.

Examples of the host, which may be employed, are bacteria belonging to the genus *Escherichia*, bacteria belonging to the genus *Bacillus*, yeast, insect cells, insects and animal cells, etc. As mentioned above, preferred are insect cells and animal cells.

Specific examples of the bacteria belonging to the genus *Escherichia* include *Escherichia coli* K12 DH1 (Proc. Natl. Acad. Sci. U.S.A., 60, 160 (1968)), JM103 (Nucleic Acids Research, 9, 309 (1981)), JA221 (Journal of Molecular Biology, 120, 517 (1978)), HB101 (Journal of Molecular Biology, 41, 459 (1969)), C600 (Genetics, 39, 440 (1954)), etc.

Examples of the bacteria belonging to the genus *Bacillus* include *Bacillus subtilis* MI114 (Gene, 24, 255 (1983)), 207-21 (Journal of Biochemistry, 95, 87 (1984)), etc.

Examples of yeast include *Saccharomyces cerevisiae* AH22, AH22R, NA87-11A, DKD-5D, 20B-12, etc.

As the insect, for example, a larva of *Bombyx mori* can be used (Maeda, et al., *Nature*, 315, 592 (1985)).

As the insect, for example, for the virus AcNPV, *Spodoptera frugiperda* cells (Sf cells), MG1 cells derived from mid-intestine of *Trichoplusia ni*, High Five<sup>TM</sup> cells derived from egg of *Trichoplusia ni*, cells derived from *Mamestra brassicae*, cells derived from *Estigmena acrea*, etc.; and for the virus BmNPV, *Bombyx mori* N cells (BmN cells), etc. are used. Examples of the Sf cell which can be used are Sf9 cells (ATCC CRL1711) and Sf21 cells (both cells are described in Vaughn, J. L. et al., *In Vivo*, 13, 213-217 (1977)).

Examples of animal cells include monkey cells COS-7, Vero, Chinese hamster cells CHO, dhfr gene deficient Chinese hamster cells CHO (hereinafter simply referred to as CHO(dhfr<sup>-</sup>) cell), mouse L cells, mouse 3T3, mouse myeloma cells, human HEK293 cells, human FL cells, C127 cells, BALB3T3 cell, Sp-2/O cells) etc.

Bacteria belonging to the genus *Escherichia* can be transformed, for example, by the method described in *Proc. Natl. Acad. Sci. U.S.A.*, 69, 2110 (1972) or *Gene*, 17, 107 (1982). Bacteria belonging to the genus *Bacillus* can be transformed, for example, by the method described in *Molecular & General Genetics*, 168, 111 (1979).

Yeast can be transformed, for example, by the method described in *Methods in Enzymology*, 194, 182-187 (1991), *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1929 (1978), etc.

Insect cells or insects can be transformed, for example, according to the method described in *Bio/Technology*, 6, 47-55 (1988), etc.

Animal cells can be transformed, for example, according to the method described in *Virology*, 52, 456 (1973).

The method of introduction to the cell of orphan receptor protein expression vector, for example, lipofection (Felgner, P.L. et al. Proc. Natl. Acad. Sci. U.S.A., 84, 7413 (1987)), Calcium phosphate (Graham, F.L. and van der Eb, A.J. Virology, 52, 456-467 (1973)), electroporation (Nuemann, E. et al. EMBO J., 1, 841-845 (1982)) are listed.

The cells capable of expressing an orphan receptor protein can be obtained using such methods.

Furthermore, as to the method of expressing an orphan receptor protein in a stable condition using animal cells, there is a method in which the cells the expression vector of which introduced to the above animal cells inserted in a gene, is selected by a clonal selection. To be more specific, using the above selection marker as an index, a transformant can be selected. For those animal cells obtained by use of the selection marker, it is possible to obtain a stable animal strain having a highly expressed orphan receptor protein by repeating the clonal selection. Moreover, using dhfr gene as a selection marker and by increasing the concentration of MTX gradually, the cells are cultured and the cell only from a residence strain is selected. It is possible to obtain the highly expression animal cell strain by amplifying the DNA encoding orphan receptor protein in the cell with dhfr gene.

Where the host is bacteria belonging to the genus Escherichia or the genus Bacillus, the transformant can be appropriately incubated in a liquid medium which contains materials required for growth of the transformant such as carbon sources, nitrogen sources, inorganic materials, and so on. Examples of the carbon sources include glucose, dextrin, soluble starch, sucrose, etc. Examples of the nitrogen sources include

inorganic or organic materials such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, meat extract, soybean cake, potato extract, etc. Examples of the inorganic materials are calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc. In addition, yeast extract, vitamins, growth promoting factors etc. may also be added to the medium. Preferably, pH of the medium is adjusted to about 5 to about 8.

A preferred example of the medium for incubation of the bacteria belonging to the genus *Escherichia* is M9 medium supplemented with glucose and Casamino acids (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972). If necessary and desired, a chemical such as 3 $\beta$ -indolylacrylic acid can be added to the medium thereby to activate the promoter efficiently. Where the bacteria belonging to the genus *Escherichia* are used as the host, the transformant is usually cultivated at about 15°C to about 43°C for about 3 hours to about 24 hours. If necessary and desired, the culture may be aerated or agitated.

Where the bacteria belonging to the genus *Bacillus* are used as the host, the transformant is cultivated generally at about 30°C to about 40°C for about 6 hours to about 24 hours. If necessary and desired, the culture can be aerated or agitated.

Where yeast is used as the host, the transformant is cultivated, for example, in Burkholder's minimal medium (Bostian, K. L. et al., Proc. Natl. Acad. Sci. U.S.A., 77, 4505 (1980)) or in SD medium supplemented with 0.5% Casamino acids (Bitter, G. A. et al., Proc. Natl. Acad. Sci. U.S.A., 81, 5330 (1984)). Preferably, pH of the medium is adjusted to about 5 to about 8. In general, the transformant is cultivated at about 20°C



to about 35°C for about 24 hours to about 72 hours. If necessary and desired, the culture can be aerated or agitated.

Where insect cells or insects are used as the host, the transformant is cultivated in, for example, Grace's Insect Medium (Grace, T. C. C., *Nature*, 195, 788 (1962)) to which an appropriate additive such as immobilized 10% bovine serum is added. Preferably, pH of the medium is adjusted to about 6.2 to about 6.4. Normally, the transformant is cultivated at about 27°C for about 3 days to about 5 days and, if necessary and desired, the culture can be aerated or agitated.

Where animal cells are employed as the host, the transformant is cultivated in, for example, MEM medium containing about 5% to about 20% fetal bovine serum (*Science*, 122, 501 (1952)), DMEM medium (*Virology*, 8, 396 (1959)), RPMI 1640 medium (*The Journal of the American Medical Association*, 199, 519 (1967)), 199 medium (*Proceeding of the Society for the Biological Medicine*, 73, 1 (1950)), etc. Preferably, pH of the medium is adjusted to about 6 to about 8. The transformant is usually cultivated at about 30°C to about 40°C for about 15 hours to about 60 hours and, if necessary and desired, the culture can be aerated or agitated.

In the case of using CHO (dhfr<sup>-</sup>) cells and dhfr gene as a selection marker, DMEM medium containing thymidine-free dialysed fetal bovine serum is preferred to use.

The cell membrane fraction refers to a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica

Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by fractionation using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in an orphan receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the receptor protein and in the membrane fraction is preferably  $10^3$  to  $10^8$  molecules per cell, more preferably  $10^5$  to  $10^7$  molecules per cell. As the amount of expression increases, the ligand binding activity per unit of membrane fraction (specific activity) increases so that not only the highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot. The cells which do not express the orphan receptor proteins or its cell membrane fraction refers to the cells listed above as a host cell, which does not express the orphan receptor proteins. As its cell membrane fraction, those same as above can be used.

(C) The test compound and test compound (a),

In this specification, "test compound" or "test compound (a)" refers to natural/non-natural peptides, natural/non-natural proteins, natural/non-natural non-peptide compounds, synthetic compounds,

naural/unnatural fermentation products as examples. Those peptides, proteins, compounds and fermentation products used for test compounds and test compound (a) can form salts. For salts of the orphan receptor protein, preferred are salts with physiologically acceptable acids, especially physiologically acceptable acid addition salts. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

(D) A cell stimulating activity:

When a test compound (more specifically, test compound (a)) is brought in contact with cells capable of expressing an orphan receptor protein or the salts thereof and when a test compound (more specifically, test compound (a)) is brought in contact with cells which are not capable of expressing orphan receptor protein or the salts thereof, each cell stimulating activity can be measured using as index, for example, (a) change in extracellular pH, (b) arachidonic acid release, (c) acetylcholine release, (d) intracellular  $\text{Ca}^{2+}$  release, (e) change in intracellular cAMP production, (f) change in intracellular cGMP production, (g) inositol phosphate production, (h) change in cell membrane potential, (i) phosphorylation of intracellular proteins, (j) activation of c-fos, (k) binding of  $\text{GTP}\gamma\text{S}$ , (l) expression of a reporter gene by a publicly known method, or using an assay kit commercially available.

Among the above methods, as a method of measuring a cell stimulating activity to be used in the screening

method or ligand identification method according to the present invention, preferred is a measuring method using as an index the change in pH outside a cell.

Specifically, cells expressing an orphan receptor protein or its cell membrane fraction, and cells which do not express the orphan receptor protein or its cell membrane fraction are first cultured on a multi-well plate, respectively. Prior to the measurement of the cell stimulating activity, the medium is replaced with fresh medium or with an appropriate non-cytotoxic buffer, followed by incubation for a given period of time in the presence of a test compound (more specifically test compound (a)). Subsequently, the cells are extracted or the supernatant is recovered and the resulting product is quantified by appropriate procedures.

Where it is difficult to detect the production of the index substance (e.g., arachidonic acid) for a cell stimulating activity due to a degrading enzyme contained in the cells, an inhibitor against such a degrading enzyme may be added prior to the assay. For detecting activities such as the cAMP production suppression activity, the baseline production in the cells is increased by forskolin or the like and the suppressing effect on the increased baseline production may then be detected.

To conduct the screening by measuring the cell stimulating activities, it requires appropriate cells capable of expressing an orphan receptor protein or its cell membrane fractions and appropriate orphan receptor protein non-expression cell or its cell membrane fraction. The cells capable of expressing an orphan receptor proteins can be either a stable or a transient expression strain.

To measure a cell stimulating activity, it is preferred to keep the concentration as high as possible within discriminating the cell specific activity in order to detect the weak activities of agonists. In this case, "High concentration" means normally  $10^{-8}\text{M}$  to  $1\text{M}$ , preferably  $10^{-6}\text{M}$  to  $10^{-2}\text{M}$ . The same compounds described in above (C) may be used as the test compounds (more specifically, test compounds (a)).

Moreover, the details of the above cell stimulating activity assay system are described as follow:

In the following descriptions (1)~(7), "test compound" refers to test compound (a).

(1) Cell stimulating activity assay system characterized by measuring the fluctuation of extracellular pH (acidification rate)

The cell stimulating activity can be measured by measuring the extracellular pH change caused by reaction of a test compound having an agonist activity and cells capable of expressing an orphan receptor protein or its cell membrane fractions, using Cytosensor (Manufactured by Molecular Device).

Described below is the detail method of measuring a cell stimulating activity of a test compound by Cytosensor for measuring the extracellular pH changes

① After cultivating the orphan receptor protein expression cells or its cell membrane fraction for about 2 to 48 hours, preferably about 5 to 24 hours (for example, in the capsule of Cytosensor device), the pH of the medium is stabilized.

Until the pH becomes stable, a medium (e.g. RPMI1640 medium with 0.1% fetal bovine serum (Prepared by Molecular Device)) is perfused.

② A test compound is brought in contact with cells capable of expressing an orphan receptor protein or the

orphan receptor protein expressed on its cell membrane fractions.

It is general for such contact to perfuse the medium containing a test compound to cells capable of expressing an orphan receptor protein or its cell membrane fractions.

③ Then, the pH change in the medium when a medium containing a test compound is brought in contact with the cells capable of expressing an orphan receptor protein or an orphan receptor protein expressed on its cell membrane fractions, is measured.

④ The above methods ① to ③ using the cells which are not expressing the orphan receptor protein or its cell membrane fractions, are conducted.

(2) Cell stimulating activity assay system  
characterized by measuring the radioactivities of GTP $\gamma$ S

When a test compound having an agonist activity stimulates cells capable of expressing an orphan receptor protein, an intercellular G-protein becomes active and, as a result, GTP bonds. The same phenomena can be observed with a cell membrane of an orphan receptor protein expression. Generally, GTP is converted to GDP by hydrolysis. When GTP $\gamma$ S is added to the reaction solution, GTP $\gamma$ S bonds with G-protein (same as GTP does), and it does not suffer from hydrolysis with keeping the condition of bonding to the cell membrane containing the G-protein. Using the labeled GTP $\gamma$ S, it is possible to measure the receptor expression cell stimulating activity of the test compound by measuring the radioactivity remaining in the cell membrane. Applying this reaction, a stimulating activity of a test compound with respect to cells capable of expressing an orphan receptor protein and the orphan receptor protein non-expression cells.

This method is an assay using an orphan receptor protein and cells containing the cell membrane fractions of cells which are not capable of expressing the orphan receptor, and is an assay to measure a cell stimulating activity. In this assay, a substance which shows an activity to promote binding GTP $\gamma$ S to an orphan receptor protein cell membrane, and which shows no activity to promote binding of GTP $\gamma$ S to a cell membrane which does not express an orphan receptor protein, may be a ligand candidate substance.

The detail description for measuring a cell stimulating activity by this method is as follows.

- ① A cell membrane fraction containing an orphan receptor protein is diluted with a membrane dilution buffer (e.g. 50mM Tris, 5mM MgCl<sub>2</sub>, 150mM NaCl, 1  $\mu$ M GDP, 0.1% BSA pH7.4).
- The dilution rate may vary according to the amount of receptor protein expression.
- ② The solution obtained from Step ① is transferred partially to an appropriate container (e.g. Falcon 2053). A test compound is added to the container, and then [<sup>35</sup>S]GTP $\gamma$ S is added to make the final concentration to become 200pM.
- ③ After the medium obtained from Step ② is kept at 25°C for an hour, a relief solution for washing (e.g. ice-cooling 50mM Tris, 5mM MgCl<sub>2</sub>, 150mM NaCl, 0.1% BSA, 0.05% CHAPS pH7.4 1.5ml) is added. Then, the solution is filtered with, for example, a glass fiber filtering paper GF/F.
- ④ The filtering paper is kept at 65°C for 30minutes for drying on a liquid scintillation counter. The radioactivities of [<sup>35</sup>S]GTP $\gamma$ S in membrane fraction left on the filtering paper is measured.

- ⑤ Using a cell membrane fraction which does not contain an orphan receptor protein, the steps ① to ④ are conducted.

(3) Cell stimulating activity measurement assay system characterized by measuring change in intracellular cAMP

Cells capable of expressing an orphan receptor protein change amount of intracellular cAMP by the agonist stimuli of the test compounds containing an agonist activity. Using this reaction, the cell stimulating activities of the test compound against the cells capable of expressing an orphan receptor protein can be measured.

Using the anti-cAMP antibody obtained by immunized mice, rats rabbits goats and cows and  $^{125}\text{I}$  labeled cAMP (both are commercially available), the amount of cAMP production for the varieties of animal cells expressed an orphan receptor protein can be measured by RIA or the other EIA system such as the combination of anti-cAMP antibody and the labeled cAMP.

It is possible to conduct an assay like SPA method using beads containing the scintillant to which an anti-cAMP is fixed using an antibody to an animal IgG used for production of Protein A or an anti-cAMP antibody, and  $^{125}\text{I}$  labeled cAMP (for example, using the kit manufactured by Amasham pharmacia Biotech).

Using this assay system, it is possible to measure cAMP production promoting activity. Also, it is possible to measure a cell stimulating activity (cAMP production inhibition activity) by measuring increase amount of intercellular cAMP using a substance which causes increase in the amount of intercellular cAMP such as Forskolin, and observing the amount of intracellular cAMP when a test compound is added.

The detail description for measuring a cell stimulating activity by this method is as follows.



- ① Animal cells (e.g. CHO cell) expressing an orphan receptor protein are placed in a 24-well plate, and cultivated with an appropriate concentration ( $5 \times 10^4$  cell/well) for about 48 hours.
- ② The animal cells expressing the orphan receptor protein is washed with a buffer solution (e.g. Hanks' buffer containing 0.2mM 3-isobutyl-methylxanthine, 0.05% BSA and 20mM HEPES (pH7.4), hereinafter referred to as reaction buffer).
- ③ An appropriate amount (e.g. about 0.5ml) of the reaction buffer is added to the cell, and kept in an incubator for 30 minutes.
- ④ Then, the reaction buffer is removed from the system, and an appropriate amount (e.g. about 0.25ml) of fresh reaction buffer is added to the cell. Then, an appropriate amount (e.g. about 0.25ml) of the reaction buffer (to measure the cAMP production inhibition activity, it prefers to use the buffer containing  $2 \mu\text{M}$  forskolin) with an appropriate amount (e.g. 1nM) of the test compound are added to the cell. The solution is reacted at  $37^\circ\text{C}$  for 24 minutes.
- ⑤ 20% of perchloric acid is added to stop the reaction. Then, by placing it on ice, the intracellular cAMP is extracted.
- ⑥ The amount of cAMP in the extraction is measured by using cAMP EIA kit (Amasham pharmacia biotech).
- ⑦ The Steps ① to ⑥ are conducted with the cell which does not express the orphan receptor protein.

(4) Cell stimulating activity assay system  
characterized by introducing CRE-reporter gene

The DNA containing CRE (cAMP response element) is inserted into the multi-cloning site which is upstream of luciferase gene of Picagene basic vector

or Picagene enhancer vector (Toyo Ink). It named as CRE-reporter gene vector.

In the cell transfected by CRE-reporter gene vector, a stimulus with the increase of cAMP, induces an expression of luciferase gene through CRE and a production of luciferase protein. By measuring the luciferase activity, it is possible to detect the change in the amount of cAMP in CRE-reporter gene vector induction cells.

A cell stimulating activity can be measured using the cells capable of expressing an orphan receptor protein, to which CRE-reporter gene vector is transfected.

The detail description for the method of measuring a cell stimulating activity is as follows.

- ① Cells capable of expressing an orphan receptor to which the CRE-reporter gene is introduced, is placed in a 24-well plate with an appropriate concentration ( $5 \times 10^3$  cell/well), and cultivated for about 48 hours.
- ② The cells are washed with an appropriate amount (e.g. 0.2mM) of buffer solution (e.g. Hanks' buffer containing (pH7.4) 3-isobutyl-methyl xanthine, 0.05% BSA and 20mM HEPES, hereinafter, Hanks' buffer containing (pH7.4) 0.2mM 3-isobutyl-methyl xanthine, 0.05% BSA and 20mM HEPES, is referred to as reaction buffer).
- ③ An appropriate amount (e.g. about 0.5ml) of the reaction buffer to the cells. Then, the mixture is kept warm in a cultivator for 30 minutes.
- ④ Then, the reaction buffer is removed from the system. An appropriate amount (e.g. about 0.25ml) of the reaction buffer is added to the cells. Then, an appropriate amount (e.g. about 0.25ml) of the reaction buffer (to measure the cAMP production inhibition activity, it prefers to use the buffer

containing 2  $\mu$ M forskolin) and an appropriate amount (e.g. 1nM) of the test compounds are added to the cells. The reaction is carried out at 37°C for 24 hours.

- ⑤ The cells are dissolved in a cell lysis solution for Picagene (Toyo Ink). To the cell lysate, a luminescent substance (Toyo Ink) is added.
- ⑥ The luminescence is measured by a luminometer, a liquid scintillation counter, a top counter or the like.
- ⑦ The steps ① to ⑥ are conducted with the cells which do not express the orphan receptor protein or its cell membrane fraction.

Alkaline phosphatase, chloramphenicol, acetyltransferase or  $\beta$ -galactosidase can be used as a reporter gene, besides luciferase. The activity of the gene production of reporter gene can be measured easily using commercially available measuring kit. That means, the activity of alkaline phosphatase can be measured by Lumi-Phos 530 (Wako); the activity of chloramphenicol and acetyltransferase can be measured by FAST CAT Chrolamphenicol Acetyltransferase Assay Kit (Wako); and the activity of  $\beta$ -galactosidase can be measured by Aurora Gal-XE (Wako).

(5) A cell stimulating activity assay system characterized by measuring arachidonic acid release

The cells capable of expressing an orphan receptor protein release the metabolic substance of arachidonic acid to outside of the cells due to the stimulus by an agonist. If arachidonic acid having radio activity is taken into the cell beforehand, it is possible to measure a cell stimulating activity by measuring the radioactivities released outside the cells. In this process, if the test compound is

added, it is possible to measure a cell stimulating activity by checking the arachidonic acid metabolite release activity of the test compound.

The detail description for the method of measuring a cell stimulating activity is as follows.

- ① Cells capable of expressing an orphan receptor are placed in a 24-well plate, and cultivated with an appropriate concentration ( $5 \times 10^4$  cell/well) for about 24 hours.
  - ② After cultivation, an appropriate amount ( $0.25 \mu$  Ci/well) of [ $^3$ H] arachidonic acid is added. 16 hours after adding [ $^3$ H] arachidonic acid, the cells are washed with a washing solution (Hanks' buffer (pH7.4) containing 0.05% BSA and 20mM HEPES). Then, the test compound dissolved in a buffer solution (e.g., Hanks' buffer (pH7.4) containing 0.05% BSA and 20mM HEPES, hereinafter, Hanks' buffer (pH7.4) containing 0.05% BSA and 20mM HEPES is referred to as reaction buffer) is added to each well.
  - ③ After incubating at 37°C for 60 minutes, an appropriate amount of the reaction buffer (e.g.  $400 \mu$  l) is added to a scintillator. Then, the amount of [ $^3$ H] arachidonic acid metabolite is measured by a scintillation counter.
  - ④ The steps ① to ③ are conducted with the cells which do not express an orphan receptor protein.
- (6) A cell stimulating activity assay system, which is characterized by measuring intracellular  $\text{Ca}^{2+}$  release
- When a test compound having an agonist activity stimulates cells which are expressing an orphan receptor, the intracellular  $\text{Ca}^{2+}$  concentration increases. Using this fact, the cell stimulating activity can be measured.
- ① Cells capable of expressing an orphan receptor protein are placed on a sterilized cover glass for a

microscope. After about 2 days, the medium is replaced with HBSS in which an appropriate amount (4mM) of Fura-2 AM (Dojin Kagaku) is suspended, and left for 2 and half hours at room temperature.

- ② After washing with HBSS, a cover glass to a cuvet is set. The increase in the ratio of intensity of fluorescence at 505nm where the excitation wave length is 340nm and 380nm, is measured by a spectrophotofluorometer when the test compound is added.

FLIPR (Manufactured by Molecular device) can be also used as follows.

That is, ① Fluo-3 AM (Manufactured by Dojin Kagaku) is added to the cell suspension to let the cell comprise Fluo-3AM. The supernatant is washed by centrifuging several times, and placed in a 96-well plate. ② The cells are set to FLIPR, and a test compound is added in the same way as Fura-2. The change in the intensity of the fluorescence observed when the test compound is added, is measured. Also, a cell stimulating activity can be measured by allowing cells capable of expressing an orphan receptor protein to co-express a protein gene such as Aequorin which emits light by the increase of the intercellular calcium, considering the fact that the increase of the intercellular calcium ion causes Aequorin to become calcium binding type and it emits light as a result, and measuring the change in the intensity of luminescence observed when the test compound is added. This method is almost same as above except that this method does not require the cell to take in the fluorescence substance itself.

To measure the change in the intercellular calcium ion concentration more easily, it is possible to use cells which expresses a modified G-protein such as chimera G-protein at the same time. Said chimera G-

protein refers to a G-protein in which a G-protein such as  $G_i$ ,  $G_o$ ,  $G_s$  or the like, which does not use  $Ca^{2+}$  in a signal transduction system is substituted by the functional domain of G-protein such as  $G_9$ ,  $G_{11}$  or the like, which uses  $Ca^{2+}$  in a signal transduction system. By using said chimera protein, the signal transmission of any G-protein can be monitored through  $Ca^{2+}$  change.

③ The steps ① to ② are conducted with using cells which are not capable of expressing an orphan receptor protein.

(7) A cell stimulating activity assay system, which is characterized by measuring inositol phosphate production

By adding a test compound having an agonist activity to cells capable of expressing an orphan receptor protein, the concentration of inositol triphosphate rises. By observing the reaction for cells capable of expressing orphan receptor protein caused by the test compound, a cell stimulating activity can be measured.

① The detail description for the method of measuring a cell stimulating activity is as follows. Cells capable of expressing an orphan receptor are placed in a 24-well plate, and left for one day, and cultivated for one day in a medium in which myo-[2- $^3H$ ]inositol ( $2.5 \mu Ci/well$ ) is added. After washing cells with the medium, a test compound is added thereto and 10% Perchloric acid is added to stop the reaction.

② The reaction solution is neutralized with an appropriate amount of KOH (e.g. 1.5M) and an appropriate amount of HEPES solution (60mM). The cells are passed through a column filled with AG1x8 resin (Bio Rad). After washing, the radioactivities, which is eluted by an appropriate amount of  $HCOONH_4$  (e.g. 1M) and an appropriate

amount of HCOOH (e.g. 0.1M) is measured by a liquid scintillation counter.

- ③ The steps ① to ② are conducted with using cells which are not capable of expressing an orphan receptor protein.

(E) (Test) compounds having an agonist activity

In this specification, "a (test) compound containing an agonist activity" means that a test compound described in above (c) (e.g., natural/non-natural peptide, natural/non-natural protein, natural/non-natural non-peptide, synthetic compounds and natural/unnatural fermentation products) or test compound (a), which shows a cell stimulating activity when it is brought into contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions in any one of the above cell stimulating activity assay systems (preferably, a cell stimulating activity assay system characterized by measuring the change in the extracellular pH (acidification rate)) and when a cell stimulating activity is not observed in the case of using cells which are not capable of expressing an orphan receptor protein or its cell membrane fractions (e.g. natural/non-natural peptide, natural/non-natural protein, natural/non-natural non-peptide, synthetic compounds and natural/unnatural fermentation products as examples).

Moreover, examples of test compounds having an agonist activity when an orphan receptor protein is FM-3 (Tan, C.P. et al., Genomics 52, 223-229, 1998), are peptides containing C-terminus R-X-NH<sub>2</sub> structure (X indicates amino acid residue such as Gly, Ala, Val, Leu, Ile, Ser, Thr, Cys, Met, Glu, Asp, Lys, Arg, His, Phe, Tyr, Trp, Pro, Asn, Gln etc.), more specifically,

peptides having the amino acid sequence represented by SEQ ID NO:2, 6 and 20. The peptides, protein, compounds and fermentation product which are test compounds having an agonist activity can form salts. These salts can be salts with physiologically acceptable bases (e.g., alkali metal) or acids (organic acids, inorganic acids). Physiologically acceptable acid addition salts are preferred. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

As to the cell stimulating activity assay systems according to the above (D), the judgement standard to determine whether a test compound (or test compound (a)) is a (test) compound having an agonist activity or not, is described as follows. However, this standard is just an example, and thus it is not used to limit the interpretation as to whether a test compound (or the test compound (a)) has an agonist activity or not.

In the case of using the cell stimulating activity assay system which characterized by measuring a change in extracellular pH (acidification rate) according to above (D)-(1), when an extracellular pH is over 105% when a test compound is brought in contact with a cell (where an extracellular pH before a test compound is brought to be in contact with the cell is set as 100%), and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cell or its cell membrane fractions, such test compound is selected as the (test) compound having an agonist activity.



In the case of using a cell stimulating activity assay system which is characterized by measuring the radioactivities labeled GTP $\gamma$ S, when a radio-activation of a test region is over 105% when a test compound is added (where a radioactivities without adding a test compound is set as 100%) and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cell or its cell membrane fractions, such compound is selected as a (test) compound having an agonist activity.

In the case of using a cell stimulating activity assay system which characterized by measuring the change in the intracellular cAMP as described in (D)-(3), when the amount of produced cAMP is over 105% in the case of adding a test compound (where the amount of produced cAMP by forskolin stimulation is set as 100% in the case of using the cAMP production inhibition function as an index), and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cell or its cell membrane fractions, such test compound is selected as a (test) compound having an agonist activity. On the other hand, in the case of setting cAMP production promoting function as an index, when the amount of produced cAMP is over 105% in the case of adding a test compound (where the amount of cAMP without adding a test compound is set as 100% as an index), and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a (test) compound having an agonist activity.

In the case of using a cell stimulating activity assay system characterized by inducing the CRE-reporter gene described in above (D)-(4), (when the

cAMP production inhibition function is set as an index and where the luminescence occurred by the forskolin reaction is set as 100%), when the amount of luminescence is less than 95% and when a stimulating activity is not observed in the case of using an orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a (test) compound having an agonist activity. On the other hand, (when the cAMP production promoting function is set as an index and where the amount of luminescence is set as 100% when a test compound is not added), when the amount of luminescence is over 105% and when a stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such compound is selected as the (test) compound having an agonist activity. In the case of using a cell stimulating activity assay system characterized by measuring the arachidonic acid release described in above (D)-(5), (where the amount of [ $H^3$ ] arachidonic acid metabolite in a medium by a test compound non-adding reaction buffer is set as 100%), when the amount of [ $H^3$ ] arachidonic acid metabolite in the medium in which a test compound is added is over 105% and when a cell stimulating activity is not observed in the case of using an orphan receptor protein non-expression cell or its cell membrane fractions, such test compound is selected as a (test) compound having an agonist activity.

In the case of using a cell stimulating activity assay system characterized by measuring the  $Ca^{2+}$  release described in above (D)-(6), (where the intensity of luminescence without adding the test compound is set as 100%), when the intensity of luminescence of the compound which the test compound

added is over 105% and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fractions, such test compound is selected as a (test) compound having an agonist activity.

In the case of using a cell stimulating activity assay system characterized by measuring the production of inositol phosphate described in above (D)-(7), (where a radioactivity in a medium by a test compound non-adding reaction buffer is set as 100%), when a radioactivity in a medium which a test compound is added is over 105% and when a cell stimulating activity is not observed in the case of using orphan receptor protein non-expression cells or its cell membrane fraction, such test compound is selected as a (test) compound having an agonist activity.

(F) Structural comparison of a (test) compound having an agonist function:

After selecting (test) compounds having an agonist activity, by comparing the structures of the (test) compounds, a common structure of the (test) compounds is estimated (or determined) to obtain or prepare a ligand candidate substance of said common structure.

Said ligand candidate substance refers to a compound having a common structure with the test compounds having an agonist activity and a substance having a stronger cell stimulating activity of above (D) as comparing with each test compound (more specifically, test compound (a)) (e.g. natural peptide, natural protein and natural non-peptide compound).

In the case where (test) compounds having an agonist activity are natural/non-natural peptide or natural/non-natural protein, the amino acid sequences encoding the peptides or proteins are compared, and then the common structure is considered to be a partial

sequence which is highly homologous or a part having a similar steric structure.

More specifically, for example, when FM-3 is an orphan receptor protein, is as a common structure, by comparing the SEQ ID NO: 2,6,20, the fact that "R-X-NH<sub>3</sub> structure at C-terminus ("X" represents the voluntary amino acid residue)" is a common structure for FM-3 can be induced. From said common structure, the method of screening or the method of determination of a ligand of the present invention, when FM-3 is the orphan receptor protein, it is considered to be the ligand candidate substance containing a common structure, moreover, the ligand of FM-3 (endogenous) is the peptide containing the structure of "R-X-NH<sub>3</sub>" at C-terminus.

As the peptide containing the structure of "R-X-NH<sub>3</sub>", A-18-F-NH<sub>2</sub>, F-8-F-NH<sub>2</sub>, (Perry, S. J. et al. FEBS Lett. 409: 426-430, 1997), prolactin-releasing peptide (Hinuma, S, et al. Nature 393: 272-276, 1998) are known in mammal. For lower animals, there are groups of RF amino-peptide family universally so, it is possible that there might be FM-3 (endogenous) ligand in a novel "R-X-NH<sub>3</sub>" peptide.

If the (test) compounds having an agonist activity are a natural/non-natural non-peptide compound or a synthetic compound, the chemical structures of those compounds are compared, to identify a basic structure (e.g. specific cyclic structures, for example, cycloalkyl as "alicyclic hydrocarbon", cycloalkenyl, cycloalkandienyl and the like as saturated or unsaturated alicyclic hydrocarbon, aromatic heterocycle as "heterocycle", saturated or unsaturated non-aromatic heterocycle (alifatic heterocycle) etc.) as a common structure.

Also, since the identification of a ligand of the present invention is made using the above common

structure as an index, it is much easier and much more certain to obtain a subtype of ligand having structural similarity with the ligand as compared with a current method.

(G) A process after obtaining or preparing a ligand candidate which is identified by considering a common structure of the test compound containing an agonist activity:

A method of obtaining or preparing a ligand candidate substance which is identified by considering a common structure of the test compound containing an agonist activity, is described as follows.

(1) A method of preparing or obtaining a ligand candidate substance by selecting a ligand candidate substance having a common structure by searching a natural peptide, a natural protein and a natural non-peptide compound having a common structure

A ligand and its subtype can be estimated by searching a natural peptide, a natural protein and a natural non-peptide compound containing a common structure based on a common structure of test compounds (more specifically, test compound (a)) described in above (F).

There are quite a few publicly know databases which can be used. The representative databases are, for example, Beilstein Handbook of Organic Chemistry (Beilstein), CROPR (Serwent Crop Protection Registry) file (Derwent), Derwent Drug file (Derwent) and the Merck Index (Merck).

Then, for those estimated ligand, it is possible to determine whether or not it is a ligand and its subtype by measuring its cell stimulating activity, and comparing it with a cell stimulating activity of a ligand candidate substance using the cell stimulating activity assay system described in above (D).

(2) A method of obtaining a ligand candidate substance by cloning cDNA or a gene encoding a ligand and its subtype by preparing a primer or a probe having a base sequence encoding a common structure (where the test compound having an agonist activity is a peptide or a protein).

First, a primer or probe is prepared, which has a common structure of (test) compounds having an agonist activity described in above (F), that is, a base sequence encoding a partial sequence which is highly homologous to the amino acid sequence encoding the (test) compounds having an agonist activity.

Amplification is made, by known PCR techniques using the primer or probe, to a DNA encoding a ligand candidate substance derived from any tissues (e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract, blood vessel, heart, etc.) of human, other warm-blooded animals (e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.) and fish, or a genomic library and cDNA library derived from cells.

The cDNA comprising the base sequence encoding a cloned ligand candidate substance can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may contain ATG as a translation initiation codon at the 5' end thereof and TAA, TGA or TAG as a translation termination codon at the 3' end thereof. These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

According to the preparation method for a cells capable of expressing an orphan receptor protein

described in above (A) and (B), a transformant containing the DNA encoding a ligand candidate substance is cultured. It is possible to separate and purify the ligand candidate substance from the cultivated product.

After cultivation, the transformant or cell is collected by a publicly known method and suspended in a appropriate buffer. The transformant or cell is then disrupted by publicly known methods such as (ultra)sonication, a treatment with lysozyme and/or freeze-thaw cycling, followed by centrifugation, filtration, etc. Thus, the crude extract of the estimated ligand or its subtypes can be obtained. The buffer used for the procedures may contain a protein modifier such as urea or guanidine hydrochloride, or a surfactant such as Triton X-100<sup>TM</sup>, etc.

The supernatant or the ligand candidate substance contained in the extract thus obtained can be purified by appropriately combining the publicly known methods for separation and purification. Such publicly known methods for separation and purification include a method utilizing difference in solubility such as salting out, solvent precipitation, etc.; a method mainly utilizing difference in molecular weight such as dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, etc.; a method utilizing difference in electric charge such as ion exchange chromatography, etc.; a method utilizing difference in specific affinity such as affinity chromatography, etc.; a method utilizing difference in hydrophobicity such as reverse phase high performance liquid chromatography, etc.; a method utilizing difference in isoelectric point such as isoelectrofocusing electrophoresis; and the like.

When the ligand candidate substance thus obtained is in a free form, it can be converted into the salt by publicly known methods or modifications thereof. On the other hand, when the polypeptide is obtained in the form of a salt, it can be converted into the free form or in the form of a different salt by publicly known methods or modifications thereof.

The ligand candidate substance can be prepared by publicly known methods for peptide synthesis, or by cleaving the ligand candidate substance with an appropriate peptidase. For the methods for protein synthesis, for example, either solid phase synthesis or liquid phase synthesis may be used. That is, the partial peptide or amino acids that can construct the polypeptide of the present invention are condensed with the remaining part of the partial peptide of the present invention. Where the product contains protecting groups, these protecting groups are removed to give the desired peptide. Publicly known methods for condensation and elimination of the protecting groups are described in 1) - 5) below.

1) M. Bodanszky & M.A. Ondetti: Peptide Synthesis, Interscience Publishers, New York (1966)

2) Schroeder & Luebke: The Peptide, Academic Press, New York (1965)

3) Nobuo Izumiya, et al.: *Peptide Gosei-no-Kiso to Jikken* (Basics and experiments of peptide synthesis), published by Maruzen Co. (1975)

4) Haruaki Yajima & Shunpei Sakakibara: *Seikagaku Jikken Koza* (Biochemical Experiment) 1, *Tanpakushitsu no Kagaku* (Chemistry of Proteins) IV, 205 (1977)

5) Haruaki Yajima ed.: *Zoku Iyakuhin no Kaihatsu* (A sequel to Development of Pharmaceuticals), Vol. 14, Peptide Synthesis, published by Hirokawa Shoten



After completion of the reaction, the product may be purified and isolated by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography and recrystallization to give the ligand candidate substance. When the ligand candidate substance obtained by the above methods is in a free form, the peptide can be converted into an appropriate salt by a publicly known method; when the protein is obtained in a salt form, it can be converted into a free form or a different salt form by a publicly known method.

Using the assay system of a cell stimulating activity described in above (d) for a ligand candidate substance, it is possible to find out whether the ligand candidate substance has an agonist (ligand) activity by measuring a cell stimulating activity and comparing it with that of a test compound (more specifically, test compound (a)). It is possible to find out whether a ligand candidate substance the agonist (ligand) activity of which is observed, is a (endogenous) ligand, its subtype for an orphan receptor protein or not, by the ligand identification method described later in (J).

Moreover, mRNA of the desired gene is purified using the probes such as Gene Trapper to acquire cDNA from the mRNA purified. Further, according to the preparation method for the orphan receptor protein described in above (A), it is possible to obtain a ligand candidate compound by culturing the transformant, which contain the DNA encoding the ligand candidate substance.

(3) A method of searching a ligand candidate substance by searching sequence database for a peptide or a

protein having a common structure (when a (test) compound is a peptide, a protein or their salt)

A ligand candidate substance can be identified by searching a common structure of a (test) compound having an agonist activity described in above (F) (i.e., a protein or a peptide containing the base sequence encoding the partial sequence which is highly homologue to the amino acid sequence encoding a (test) compound having an agonist activity) in database.

Sequence databases are, for example, GenBank (registered trade mark) file (National Institute of Health), VTS (virtual transcribed sequence).

When the base sequence of a ligand candidate substance is once identified, it is possible to obtain a ligand candidate substance according to the method described in above (G)-2.

Moreover, as a result of searching database, when the sequence which is supposed to encode a part of ligand candidate substance, is discovered, it is possible to obtain a ligand candidate substance by preparing a primer or a probe based on said sequence and in accordance with the method described in above (G)-2,.

(4) A method of searching a ligand candidate substance by preparing an antibody which recognizes a common structure

A common structure of a (test) compound having an agonist activity described in above (F) (i.e., a peptide represented by a partial sequence which is homologues to the amino acid sequence encoding a (test) compound having an agonist activity) is prepared according to the synthesis methods for preparing peptides (proteins) as described above.

Then, an antibody to said peptide is prepared according to the following method. Antibodies to the peptide may be any of polyclonal antibodies and monoclonal antibodies, as long as they are capable of recognizing the peptide. The antibodies to the peptide may be prepared by publicly known methods for preparing antibodies or antisera, using as antigens the peptide.

[Preparation of monoclonal antibody]

(a) Preparation of monoclonal antibody-producing cells

The receptor protein of the present invention is administered to mammals either solely or together with carriers or diluents to the site where the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once in every two to six weeks and 2 to 10 times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats and fowls, with mice and rats being preferred.

In the preparation of monoclonal antibody-producing cells, warm-blooded animals, e.g., mice, immunized with an antigen wherein the antibody titer is noted is selected, then the spleen or lymph node is collected after 2 to 5 days from the final immunization and antibody-producing cells contained therein are fused with myeloma cells to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may be made, for example, by reacting a labeled form of the peptide, which will be described later, with the antiserum followed by assaying the binding activity of the labeling agent bound to the antibody. The fusion may be operated, for example, by the known Koehler and Milstein method (Nature, 256, 495,

1975). Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc., of which PEG is preferably employed.

Examples of the myeloma cells are NS-1, P3U1, SP2/0, AP-1, etc. In particular, P3U1 is preferably employed. A preferred ratio of the count of the antibody-producing cells used (spleen cells) to the count of myeloma cells is within a range of approximately 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of approximately 10 to 80% followed by incubating at about 20 to about 40°C, preferably at about 30 to about 37°C for about 1 to about 10 minutes, an efficient cell fusion can be carried out.

Various methods can be used for screening of a monoclonal antibody-producing hybridoma. Examples of such methods include a method which comprises adding the supernatant of hybridoma to a solid phase (e.g., microplate) adsorbed with the peptide as an antigen directly or together with a carrier, adding an anti-immunoglobulin antibody (when mouse cells are used for the cell fusion, anti-mouse immunoglobulin antibody is used) labeled with a radioactive substance or an enzyme, or Protein A and detecting the monoclonal antibody bound to the solid phase, and a method which comprises adding the supernatant of hybridoma to a solid phase adsorbed with an anti-immunoglobulin antibody or Protein A, adding the receptor protein labeled with a radioactive substance or an enzyme and detecting the monoclonal antibody bound to the solid phase.

The monoclonal antibody can be selected by publicly known methods or by modifications of these methods. In general, the selection can be effected in a medium for animal cells supplemented with HAT (hypoxanthine, aminopterin and thymidine). Any

selection and growth medium can be employed as far as the hybridoma can grow therein. For example, RPMI 1640 medium containing 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium (Wako Pure Chemical Industries, Ltd.) containing 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku Co., Ltd.) and the like can be used for the selection and growth medium. The cultivation is carried out generally at 20°C to 40°C, preferably at about 37°C, for 5 days to 3 weeks, preferably 1 to 2 weeks. The cultivation can be conducted normally in 5% CO<sub>2</sub>. The antibody titer of the culture supernatant of hybridomas can be determined as in the assay for the antibody titer in antisera described above.

(b) Purification of monoclonal antibody

Separation and purification of a monoclonal antibody can be carried out by methods applied to conventional separation and purification of immunoglobulins [e.g., salting-out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (e.g., DEAE), ultracentrifugation, gel filtration, or a specific purification method which comprises collecting only an antibody with an activated adsorbent such as an antigen-binding solid phase, Protein A, Protein G, etc. and dissociating the binding to obtain the antibody].

[Preparation of polyclonal antibody]

The polyclonal antibody of peptide above can be manufactured by publicly known methods or modifications thereof. For example, a complex of immunogen (peptide antigen) and a carrier peptide is prepared, and a mammal is immunized with the complex in a manner

similar to the method described above for the manufacture of monoclonal antibodies. The product containing the antibody to the receptor protein of the present invention is collected from the immunized animal followed by separation and purification of the antibody.

In the complex of an immunogen and a carrier protein used to immunize a mammal, the type of carrier peptide and the mixing ratio of a carrier to hapten may be any type and in any ratio, as long as the antibody is efficiently produced to the hapten immunized by crosslinking to the carrier. For example, bovine serum albumin, bovine thyroglobulins, keyhole limpet hemocyanin, etc. is coupled to hapten in a carrier-to-hapten weight ratio of approximately 0.1 to 20, preferably about 1 to about 5.

A variety of condensing agents can be used for the coupling of a carrier to hapten. Glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group, etc. are used for the coupling.

The condensation product is administered to warm-blooded animals either solely or together with carriers or diluents to the site in which the antibody can be produce by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvant or incomplete Freund's adjuvant may be administered. The administration is usually made once approximately in every 2 to 6 weeks and about 3 to about 10 times in total.

The polyclonal antibody can be collected from the blood, ascites, etc., preferably from the blood of mammals immunized by the method described above.

The polyclonal antibody titer in antiserum can be assayed by the same procedure as that for the

determination of serum antibody titer described above. The separation and purification of the polyclonal antibody can be carried out, following the method for the separation and purification of immunoglobulins performed as applied to the separation and purification of monoclonal antibodies described hereinabove.

Thus, using the cross-linking reaction for the antigen recognizing the structure of the obtained compound having an agonist activity, a ligand candidate substance can be detected. And using the varieties of extraction methods and chromatography, the ligand candidate can be obtained using the combination of the varieties of extraction methods and chromatography.

(H) Screening method for a compound which promotes or inhibits a function of a orphan receptor protein:

It is possible to conduct a screening of a compound which promotes a function of an orphan receptor protein (highly active agonist) or a compound (agonist) which inhibits a function of an orphan receptor.

A compound which promotes a function of an orphan receptor protein is described as "highly active agonist". The term "highly active" means that it shows a stronger cell stimulating activity (moreover, a cell stimulating activity described above (D)) as comparing to "the (test) compound having an agonist activity" described in above (E).

The screening method is described bellow.

It is possible to effectively conduct screening of a compound which alters binding level between the ligand candidate substance obtained in above (G) and an orphan receptor protein (e.g. peptide, protein, non-peptide compound, synthetic compound, fermentation product, etc.) or its salts, using an orphan receptor protein or a receptor binding assay using an expression

system to be constructed using a constructed recombinant orphan receptor protein expression system.

That is, cell stimulating activities as described in above (D) such as (a) changes in extracellular pH, (b) arachidonic acid release, (c) acetylcholine release (d) intracellular  $\text{Ca}^{2+}$  release, (e) changes in intracellular cAMP (level), (f) changes in intracellular cGMP (level), (g) inositol phosphate production, (h) changes in cell membrane potential, (i) phosphorylation of intracellular proteins, (j) activation of c-fos, (k) GTP  $\gamma$  S bonds (l) expression of reporter gene as an index, are measured when an orphan receptor protein expression cells or its cell membrane fractions is brought in contact with a ligand candidate substance and when it is brought in contact with a test compound (b) (that is, when said orphan receptor protein expression cells or its cell membrane fractions is brought in contact with a compound of a candidate substance which promotes and inhibits the function of said orphan receptor protein).

If a cell stimulating activity to be measured when test compound (b) (that is, said orphan receptor protein expression cells or its cell membrane fractions to a compound of a candidate substance which accelerates and inhibits the function of said orphan receptor protein) is brought in contact with cells capable of expressing an orphan receptor protein or its cell membrane fractions as compared to a cell stimulating activity to be measured when a ligand candidate substance is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, the test compound (b) (or the candidate substance) is possible to be a compound which promotes the function of said orphan receptor protein (what is called agonist).



To find out whether said test compound (b) (or a candidate compound) is a compound which (selectively and specifically) promotes the function of said orphan receptor protein (what is called agonist) or not, the amount of specific binding between the said orphan receptor protein and said test compound (b) (or a candidate substance) is determined.

As the method for determining the amount of specific binding, for example, there is a method of determining the amount of specific binding when a labeled test compound (or a candidate compound) is brought in contact with an orphan receptor protein. As a result, if there is a sufficient binding amount (over 1% increase, preferably 10% increase in the binding amount against non-specific binding), said test compound (b) (or a candidate substance) is recognized as a compound which promotes the function of the orphan receptor protein (what is called agonist).

The detail of said method for determination is as follows.

First, an orphan protein receptor being used for the determination can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed a large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above. It is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA fragment encoding the orphan receptor protein into

host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SR $\alpha$  promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, the subject containing an orphan receptor protein may be an orphan receptor protein purified by publicly known method, cells containing a orphan receptor protein or membrane fractions of such cells.

The cells containing the orphan receptor protein are host cells that have expressed the orphan receptor protein, which host cells include insect cells, animal cells and the like.

The cells containing an orphan receptor protein are host cells which are expressing the orphan receptor protein. Preferred are insect cells and animal cells.

The cell membrane fractions are a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by using a centrifugal force, such as centrifugation for

fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the orphan receptor protein and in the membrane fraction is preferably  $10^3$  to  $10^8$  molecules per cell, more preferably  $10^5$  to  $10^7$  molecules per cell.

As the labeled test compound (b) (or candidate compound), for example, test compound (b), which is labeled with [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc., is used.

To conduct the method for determination, first, a standard receptor preparation is prepared by suspending cells containing the orphan receptor protein or the membrane fraction thereof in a buffer appropriate for use in the determination method. Any buffer can be used so long as it does not interfere with candidate - receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80<sup>TM</sup> (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. A given amount (5,000 to 500,000

cpm) of the labeled test compound (b) is added to 0.01 ml to 10 ml of the receptor solution. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity in the glass fiber filter paper is then measured by means of a liquid scintillation counter or  $\gamma$ -counter.

On the other hand, when test compound (b) is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions and show a weak or no cell stimulating activity compared with a cell stimulating activity measured when a ligand candidate substance is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, the compound (b) (or candidate compound) could be a compound which inhibits the function of the orphan receptor protein (what is called, antagonist).

Moreover, it is possible that test compound (b) (or candidate compound) is the compound which inhibits the function of the orphan receptor protein (what is called, antagonist), where a weak or no cell stimulating activity is observed when cells capable of expressing an orphan receptor or its cell membrane fractions are brought in contact with the test compound (b) (or cells capable of expressing an orphan receptor or its cell membrane fractions with a compound of candidate substance which promotes or inhibits the function of said orphan receptor protein) or a ligand candidate substance.

To find out whether said test compound (b) (or a candidate compound) is a compound which (selectively and specifically) inhibits the function of said orphan receptor protein (what is called antagonist) or not, the amount of specific binding between the said orphan receptor protein and said test compound (b) (or a candidate substance) is measured.

As the method for determining the amount of specific binding is, for example, there is a method of measuring the amount of specific binding between a labeled test compound (or a candidate compound) and an orphan receptor protein. As a result, if there is a sufficient binding amount (over 1% increase, preferably 10% increase in the binding amount against non-specific binding), said test compound (b) (or a candidate substance) is recognized as a compound which promotes the function of orphan receptor protein (what is called antagonist).

The same method as the method for conducting screening of a compound which promotes the function of orphan receptor protein (what is called, high activation agonist) described above is used.

Moreover, it is possible to conduct screening of a compound which inhibits the function of an orphan receptor protein by measuring and comparing the amount of binding between a labeled ligand candidate substance and said orphan receptor protein when a labeled ligand is brought in contact with an orphan receptor protein and when the labeled ligand candidate compound and the test compound (b) (that is, a candidate substance of a compound which inhibits the function of an orphan receptor protein) are brought in contact with an orphan receptor protein.

The detail of said screening method is described bellow.

First, an orphan protein receptor being used for the determination can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed in a large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above. It is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA fragment encoding the orphan receptor protein into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SR $\alpha$  promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, in the above screening method, the subject containing the orphan receptor protein may be the orphan receptor protein purified by publicly known method, cells containing the orphan receptor protein or membrane fraction of such cells.

The cells containing the orphan receptor protein are host cells that have expressed the orphan receptor protein. Preferred are insect cells, animal cells and the like.

The cell membrane fractions are a fraction abundant in cell membrane obtained by a cell disruption and a subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is affected mainly by using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in an orphan receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing an orphan receptor protein and in the membrane fraction is preferably  $10^3$  to  $10^8$  molecules per cell, more preferably  $10^5$  to  $10^7$  molecules per cell. As the amount of expression increases, a ligand binding activity per unit of membrane fraction (specific activity) increases so that not only a highly sensitive screening system can be constructed but also large quantities of samples can be assayed with the same lot.

To perform screening of a compound which alters a binding property between a ligand candidate compound and an orphan receptor, an appropriate orphan receptor fraction and a labeled ligand or its subtype is required.

As a labeled ligand candidate compound, the compound labeled with [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc, is used.

More specifically, to conduct a screening of a compound which inhibits the function of an orphan receptor protein, first, cells containing an orphan receptor proteins and its cell membrane fractions are suspended with a buffer appropriate for a screening to prepare a standard receptor protein. Any buffer can be used so long as it does not interfere with ligand-receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80<sup>TM</sup> (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. To 0.01ml - 10ml of the receptor solution, a given amount (5,000 to 500,000 cpm) of labeled ligand is added, and  $10^{-4}$  M -  $10^{-10}$  M of a test compound (b) (i.e., a candidate compound which inhibits a function of an orphan receptor) is simultaneously added to be co-present. To examine non-specific binding (NSB), a reaction tube containing an unlabeled test compound in a large excess is also prepared. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to



about 24 hours, preferably about 30 minutes to about 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity on the glass fiber filter paper is then measured by means of a liquid scintillation counter or  $\gamma$ -counter. Regarding the count obtained by subtracting the amount of non-specific binding (NSB) from the count obtained in the absence of any competitive substance ( $B_0$ ) as 100%, when the amount of specific binding ( $B$ -NSB) is, for example, 50% or less, test compound (b) (what is called, the candidate substance for the compound inhibit the function of orphan receptor protein) can be selected as a the compound inhibit the function of orphan receptor protein (what is called, antagonist).

(I) The test compound (b) (what is called, the candidate substance for the compound which inhibits the function of orphan receptor protein) described in above (H), the compound which promotes or inhibits an orphan receptor protein:

As the test compound (b) (what is called, the candidate substance for the compound which inhibits the function of orphan receptor protein) described in above (H), is selected from natural/non-natural peptides, natural/non-natural proteins, natural/non-natural non-peptide compounds, synthetic compounds and natural/non-natural fermentation products.

The compound which promotes or inhibits afunction of an orphan receptor protein means a compound which is recognized as a compound which promotes afunction of an orphan receptor protein and a compound which inhibits a function of an orphan receptor protein according to the method described in above (h). The compound can form salts thereof.

Examples of the salts of the compound are physiologically acceptable bases (e.g., alkali metals) and acids (e.g., organic acids and inorganic acids). Of these salts, preferred are physiologically acceptable acid addition salts. Examples of the salts include salts with, for example, inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid and sulfuric acid); salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

Moreover, a compound which promotes a function of an orphan receptor protein (highly active agonist) and a compound which inhibits a function of an orphan receptor protein (antagonist) have a function similar to a physiological activity of a (endogenous) ligand or its subtype later described. Thus, it is useful as a safe and low toxic pharmaceutical preparation according to the ligand activity.

An antagonist against an orphan receptor protein can suppress the physiological activity of the ligand or its subtype against the orphan receptor protein. Thus, it is useful as a safe and low toxic pharmaceutical preparation which suppress the ligand activity.

A highly active agonist against an orphan receptor protein is useful as a safe and low toxic pharmaceutical preparation for increasing the physiological activity of the ligand of the orphan receptor protein.

In the case of using the antagonist and the highly active agonist obtained according to the method of the present invention as a pharmaceutical composition, a conventional means may be applied to making

pharmaceutical preparations. For example, the compound or its salts may be prepared into tablets, capsules, elixirs, microcapsules, sterile solutions, suspensions, etc.

Since the preparation thus obtained is safe and low toxic, it can be administered to human or warm-blooded animal (e.g., rat, mouse, rabbit, sheep, swine, bovine, cat, dog, monkey, etc.).

The amount of administration of the ligand or its subtype, the antagonist and the agonist may vary depending on a subject to be administered, target organ, target disease, route of administration and the like. For example, in oral administration, the dose is normally about 0.1 mg to about 100 mg, preferably about 1.0 to about 50 mg, and more preferably about 1.0 to about 20 mg per day. For non-oral administration, the single dose may vary depending on a subject to be administered, target organ, target disease, route of administration and the like. In parenteral administration, it may be intravenously administered at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, and more preferably about 0.1 to about 10 mg. For other animal species, the corresponding dose as converted per 60 kg body weight can be administered.

(J) The method of determination of the ligand of orphan receptor protein or its subtype:

The present invention provides not only the screening method for a compound which promotes or inhibits a function of an orphan receptor protein, but also, using as index a common structure of (test) compounds having an agonist activity, a method of determining a ligand of the orphan receptor protein or its subtype effectively and securely.

That is, the present invention provides a method of identifying a ligand of an orphan receptor or its subtypes by

(i) contacting a test compound with cells capable of expressing an orphan receptor or its cell membrane, to measure a cell stimulating activity mediated by said orphan receptor,

(ii) comparing the cell stimulating activities measured for each test compound, to determine a common structure of the test compounds having an agonist activity, and

(iii) measuring amount of specific binding between a ligand candidate compound having the common structure thus identified and the orphan receptor protein.

More specifically, the present invention provides a method of identifying a ligand or its subtypes, comprising:

(i) measuring cell stimulating activities to be measured when a test compound (a) is brought in contact with cells capable of expressing an orphan receptor or its cell membrane fractions, and when a test compound (a) is brought in contact with cells which are not capable of expressing an orphan receptor or its cell membrane fractions,

(ii) comparing the cell stimulating activities thus measured for each of the test compounds (a), to identify a compound(s) having an agonist activity, and

(iii) measuring the amount of specific binding between said orphan receptor protein and a ligand candidate compound which is selected by considering a common structure of the compounds having an agonist activity.

In the identification method, the same method as described above may be used with respect to a step to obtain a ligand candidate compound by (i) contacting a

test compound (more specifically compound (a)) with cells capable of expressing an orphan receptor or its cell membrane, to measure a cell stimulating activity mediated by said orphan receptor, and

(ii) comparing the cell stimulating activities measured for each test compound (more specifically compound (a)), to obtain (test) compounds having an agonist activity, to identify common structure of the test compounds having an agonist activity. Moreover, the method to determine whether said ligand candidate substance is a (endogenous) ligand or its subtype using the ligand candidate substance comprising a common structure (above), is as follows.

Whether the above ligand candidate substance is a specific ligand for an orphan receptor protein or not, it is possible to determine by measuring amount of specific binding for the ligand candidate substance to said orphan receptor protein.

That is, the method includes a method of determine the binding amount for a labeled ligand candidate substance to said orphan receptor protein when a labeled ligand candidate substance is brought in contact with the orphan receptor protein. As a result of determination, it is recognized as a ligand if there is sufficient amount of binding (over 1% increase, preferably 10% increase in the binding amount against non-specific binding).

On the other hand, when enough binding amount is not observed, said ligand candidate substance may possibly be a substance having a cell stimulating activity and a low binding amount to said orphan receptor protein, that is, a non-specific agonist-like substance.

The identification method will be described in more detail bellow.

First, an orphan protein receptor used for the said identification method can be anything as long as it contains the orphan receptor protein described above. To obtain a large volume of orphan receptor protein for screening, the orphan receptor protein which is expressed in large amount using a recombinant, is appropriate.

The orphan receptor protein can be prepared by the method described above, it is preferred to use mammalian or insect cells to express DNA encoding an orphan receptor protein. Complementary DNA is used as a fragment encoding a desired portion of protein, but this is not limitative. For example, gene fragments or synthetic DNA may also be used. For introducing a DNA fragment encoding the orphan receptor protein into host animal cells and efficiently expressing the same, it is preferred to insert the DNA fragment downstream the polyhedrin promoter of nuclear polyhedrosis virus (NPV), which is a baculovirus having insect hosts, an SV40-derived promoter, a retrovirus promoter, a metallothionein promoter, a human heat shock promoter, a cytomegalovirus promoter, an SR $\alpha$  promoter or the like. The amount and quality of the receptor expressed can be determined by a publicly known method. For example, this determination can be made by the method described in the literature (Nambi, P. et al., J. Biol. Chem., Vol. 267, pp. 19555-19559 (1992)).

Accordingly, the subject containing the orphan receptor protein may be the orphan receptor protein purified by publicly known method, cells containing the orphan receptor protein or membrane fraction of such cells.

The cells containing the orphan receptor protein mean host cells that have expressed the orphan receptor

protein. Preferred are insect cells, animal cells and the like.

The cell membrane fractions are a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by a publicly known method. Useful cell disruption methods include cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid is centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained is used as the membrane fraction. The membrane fraction is rich in the receptor protein expressed and membrane components such as cell-derived phospholipids and membrane proteins.

The amount of the orphan receptor protein in the cells containing the orphan receptor protein and in the membrane fraction is preferably  $10^3$  to  $10^8$  molecules per cell, more preferably  $10^5$  to  $10^7$  molecules per cell.

As the labeled ligand candidate substance, labeled ligand candidate substance which is labeled with [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc., is used.

To conduct the measurement method, first, a standard receptor preparation is prepared by suspending cells containing the orphan receptor protein or the membrane fraction thereof in a buffer appropriate for

screening. Any buffer can be used so long as it does not interfere with candidate-receptor binding, such buffers including a phosphate buffer or a Tris-HCl buffer having pH of 4 to 10 (preferably pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80<sup>TM</sup> (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate, may optionally be added to the buffer. Further for the purpose of suppressing the degradation of the receptor or ligand by a protease, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and pepstatin may also be added. A given amount (5,000 to 500,000 cpm) of the labeled candidate substance is added to 0.01 ml to 10 ml of the receptor solution. The reaction is carried out at approximately 0 to 50°C, preferably about 4 to 37°C for about 20 minutes to about 24 hours, preferably about 30 minutes to 3 hours. After completion of the reaction, the reaction mixture is filtrated through glass fiber filter paper, etc. and washed with an appropriate volume of the same buffer. The residual radioactivity in the glass fiber filter paper is then measured by means of a liquid scintillation counter or  $\gamma$ -counter.

In the specification and drawings, the codes of bases and amino acids are denoted in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature or by a common codes in the art, examples of which are shown below. For amino acids that may have optical isomers, L form is presented unless otherwise indicated.

DNA : deoxyribonucleic acid

cDNA : complementary deoxyribonucleic acid

A : adenine

T : thymine

G : guanine

C : cytosine



RNA : ribonucleic acid  
mRNA : messenger ribonucleic acid  
dATP : deoxyadenosine triphosphate  
dTTP : deoxythymidine triphosphate  
dGTP : deoxyguanosine triphosphate  
dCTP : deoxycytidine triphosphate  
ATP : adenosine triphosphate  
EDTA : ethylenediaminetetraacetic acid  
SDS : sodium dodecyl sulfate  
Gly : glycine  
Ala : alanine  
Val : valine  
Leu : leucine  
Ile : isoleucine  
Ser : serine  
Thr : threonine  
Cys : cysteine  
Met : methionine  
Glu : glutamic acid  
Asp : aspartic acid  
Lys : lysine  
Arg : arginine  
His : histidine  
Phe : phenylalanine  
Tyr : tyrosine  
Trp : tryptophan  
Pro : proline  
Asn : asparagine  
Gln : glutamine  
pGlu : pyroglutamic acid  
HEPES: N-[2-hydroxyethyl] piperazine-n'-[2-ethanesulfonic acid]  
HBSS: Hank's Balanced Salt Solution

The sequence identification numbers in the sequence listing of the specification indicate the following sequences, respectively.

[SEQ ID NO:1]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:2]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:3]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:4]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:5]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:6]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:7]

This shows the amino acid sequence of the sample used in Example 1, later described (see Graph 1).

[SEQ ID NO:8]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:9]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:10]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:11]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:12]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:13]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:14]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:15]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:16]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:17]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:18]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:19]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:20]

This shows the amino acid sequence of the sample used in Example 1, later described (see Table 1).

[SEQ ID NO:21]

This shows the base sequence of DNA encoding human type FM-3 (Reference Example 1, later described).

[SEQ ID NO:22]

This shows the base sequence of FM3F2 used in Reference Example 1 which later described.

[SEQ ID NO:23]

This shows the base sequence of FM3R2 used in Reference Example 1, which later described.

#### EXAMPLES

The present invention will be described in detail below with reference to REFERENCE EXAMPLES and EXAMPLES, but is not deemed to limit the scope of the present invention thereto.

REFERENCE EXAMPLE 1 Preparation of Human FM-3 expression CHO cells (see Example 1 of JP Patent Appln. No. 2000-52251).

Human FM-3 was obtained as follows. Following two kinds of synthetic DNA were synthesized according to the base sequence of human FM-3 reported in Genomics 52, 223-229 (1998).

FM3F2: 5'-GTCGACCATGGCTTGCAATGGCAGTGC GGCCAGG-3' (SEQ ID NO:22)

FM3R2: 5'-GCTAGCTCAGGATGGATCGGTCTCTTGCTG-3' (SEQ ID NO:23)

Using these synthetic DNAs, it was obtained from human fetal brain with PCR.

The reaction solution was prepared by mixing 1  $\mu$ l of cDNA solution for rat hypothalamus (0.2 ng poly (A)<sup>+</sup> RNA derived), 1  $\mu$ FM3F2 (10  $\mu$ M), 1  $\mu$ FM3R2 (10  $\mu$ M), 5  $\mu$ l of an attached 10x reaction solution, 5  $\mu$ l of dNTP (10mM), 1  $\mu$ l of Ex Taq (TaKaRa) and 36  $\mu$ l of distilled water, to make a total volume of 50  $\mu$ l.

The PCR was carried out with ThermalCycler9600. The temperature was set at 95°C for 2 minutes for denaturation, then the cycle set (98°C for 10 seconds followed by 65°C for 2 seconds and 72°C for 90 seconds) was repeated 28 times. After checking the amplification of PCR product of 1.2 kb (sized) in an

aliquot of the PCR product by electrophoresing, the PCR product was subcloned to *Escherichia coli* using TA cloning kit (Invitrogen Inc.). From *Escherichia coli* obtained from subcloning, the plasmid was extracted using plasmid extracting machine (Kurabo Co., Ltd.) and the nucleotide sequence of the insertion fragment was determined. The nucleotide sequence was matched with human type FM-3cDNA (SEQ ID NO:21) reported in literature. After cleaving by Sal I and Nhe I, the fragment of 1.2kb human type FM-3 cDNA was obtained. Moreover, the multi-cloning site of pAKKO-111H, the expression vector for animal cells, was cleaved and electrophoresed to recover the vector portion. The human type FM-3 cDNA fragment and the expression vector were ligated. Then, *Escherichia coli* JM109 was transformed to obtain *E.coli* JM 109-pAKKOFM3.

Transformant *E.coli* JM 109-pAKKOFM3 was cultured to prepare a large amount of plasmid DNA of pAKKOFM3. 20  $\mu$ g of the plasmid DNA prepared was dissolved in 1ml of physiological buffered saline (PBS), transferred into the vial of gene transfer (Wako Pure Chemical Industries, Ltd.), and shaken vigorously by a vortex mixer to form a lysosome containing DNA. CHO/dhfr<sup>-</sup> cells of  $1$  or  $2 \times 10^6$  were inoculated on cell culture dish having a diameter of 35 mm. After 20 hours of cultivation, the medium was exchanged to the new medium. The lysosome solution (25  $\mu$ l) having 0.5  $\mu$ g DNA was added to each of the dishes and incubated for 16 hours to introduce plasmid DNA into the cell. Furthermore, the medium was exchanged and cultured for one day in the new medium. The medium was exchanged to a selection medium and said cells were cultured for 3 days. Finally, said cells were treated with trypsin for digestion. The low concentration of said cells was inoculated on the selection medium (minimum essential

medium without deoxyribonucleosides and ribonucleosides, alpha medium with 10% dialyzed fetal bovine serum) to select the transformant. Only the transformants were able to survive in the medium. Thus, by subcloning repeatedly, CHO-FM3 cells were established.

EXAMPLE 1 The detection of a cell stimulating activity for FM-3 expression CHO cells using the varieties of peptide sample by site sensor assay

FM-3 expressing CHO cells were inoculated in the capsules for Cytosensor ( $2.7 \times 10^5$  cells/capsule). After incubating them overnight, the site sensor was mounted to the workstation of the site sensor. A medium for assay (low buffered RMPI 1640 medium supplemented with 0.1% bovine serum albumin), was set in the flow path of the Cytosensor, and supplied to the cells in a pump cycle of ON (80 seconds) and OFF (40 seconds). A rate change in extracellular PH (from 8 to 30 seconds after the pump stopped) was calculated as an acidification rate. A change in the acidification rate was monitored. When the acidification rate became stable, the flow path was switched to have each peptide expose to the cells for 7 minutes and 2 seconds. In the acidification rate of the each well, the data for 3 cycles immediately before the peptide exposure was standardized as 100% and the comparison of cell reactions was made. The concentration of the peptide exposed was  $1 \sim 10 \mu\text{M}$ .

Table 1 shows the result of detection of a cell stimulating activity against the peptide sample of FM-3 expression CHO cells by a site sensor assay.

(○) indicates the cases in which the acidification rate (at a peak while the reaction took place) was more than 120 %. (△) indicates the cases

in which the rate was not more than 120%, however, there was noticeable reaction. (X) indicates the cases in which there was no reaction. (?) indicates the cases in which it was hard to determine one of the above categories.

As a result, the detection of the reaction was observed in H-2980 F-8-F-NH<sub>2</sub> and Small Cardioactive Peptide B (SCPB) derived from Aplysia in FM-3 expression CHO cell. Especially, the reaction for SCPB showed the strongest reaction among all.

Table 1

| Structure   | Term                | Reaction |
|---|---------------------|----------|
| FMRfa (SEQ ID NO: 1) (Bachem)   |                     | ?        |
| YFMRFa (SEQ ID NO: 2) (Bachem)  | H-2980              | ○        |
| YGGFMRFa (SEQ ID NO: 3) (Bachem)  |                     | ?        |
| YGGFMRF (SEQ ID NO: 4) (Bachem)   |                     | ×        |
| PQRFa (SEQ ID NO: 5) (Bachem)   |                     | ×        |
| FLFQPQRFa (SEQ ID NO: 6) (Bachem)   | F-8-NH <sub>2</sub> | ○        |
| pEDPFLLRFa (SEQ ID NO: 7) (Bachem)  |                     | ×        |
| DRNFLRFa (SEQ ID NO: 8) (Bachem)  |                     | ×        |
| NRNFLRFa (SEQ ID NO: 9) (Bachem)  |                     | ?        |
| TNRNFLRFa (SEQ ID NO: 10) (Bachem)  |                     | ?        |
| PDVDHVFLRFa (SEQ ID NO: 11) (Bachem)  |                     | ×        |
| KNEFIRFa (SEQ ID NO: 12) (Bachem)   | AF-1                | ×        |
| KHEYLRFa (SEQ ID NO: 13) (Bachem)   | AF-2                | ×        |
| LPLRFa (SEQ ID NO: 14) (Peptide Ken)  |                     | ×        |
| SRAHQHSMEIRTPDINPTWYTGRGIRPVGRFa (SEQ ID NO: 15) (See PCT/JP96/03821)   | bPrRP31             | ×        |
| DPEIDPFWVYGRGVRPIGRFa (SEQ ID NO: 16) (See M.Fujimoto, et al., Biochem. Biophys. Res. Commun, 242: 436-440, 1998) | cRFa                | ×        |
| SGQSWRPQGRFa (SEQ ID NO: 17) (Bachem)   | ACEP-1              | ×        |
| LSSFVRla (SEQ ID NO: 18) (Bachem)   |                     | ×        |
| ARPGYLAFPRMa (SEQ ID NO: 19) (Bachem)   | SCPA                | ?        |
| MNYLAFPRMa (SEQ ID NO: 20) (Bachem)   | SCPB                | ○        |

The structures of the peptides the reaction of which was detected (H-2980, F-8-F-NH<sub>2</sub>, SCPB) were compared. As the common structure of all, R-X-NH<sub>2</sub> structure of C-terminus part was discovered.

FIGURE 1 indicates the comparison of a commonness of amino acid sequence. Since the reaction with SCPB was observed to be strongest, it was expected that FM-3 endogenous ligand would have the similar structure to SCPB besides C-terminus R-X-NH<sub>2</sub> structure in other parts. Moreover, it was expected that the endogeneous ligands may have the similar structure to H-2980, F-8-NH<sub>2</sub> partially. From such expectations, known peptides were searched and as a result, Neuromedine U (NMU-8) was found.

#### INDUSTRIAL APPLICABILITY

According to the present invention, a ligand or its subtype of an orphan receptor protein, antagonist and highly active agonist can be obtained effectively and securely, by contacting test compounds with cells expressing an orphan receptor protein, its cell membrane fractions or orphan receptor proteins expressed on cells expressing the orphan receptor protein or its cell membrane fractions, measuring orphan receptor protein-mediated cell-stimulating activities of the test compounds, comparing the cell stimulating activities measured for each test compound to identify agonists, and then comparing the structures of the agonists.